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A Jagged 1–Notch 4 molecular switch mediates airway inflammation induced by ultrafine particles

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Abstract

Background—Exposure to traffic-related particulate matter (PM) promotes asthma and allergic diseases. However, the precise cellular and molecular mechanisms by which PM exposure acts to mediate these effects remain unclear.

Objective—We sought to elucidate the cellular targets and signaling pathways critical for the augmentation of allergic airway inflammation induced by ambient ultra fine particles (UFP).

Methods—We employed *in vitro* cell culture assays using lung-derived antigen presenting cells and allergen-specific T cells, and *in vivo* mouse models of allergic airway inflammation that employed myeloid lineage-specific gene deletions, cellular reconstitution approaches and antibody inhibition studies.

Results—We identified lung alveolar macrophage (AM) as the key cellular target of UFP in promoting airway inflammation. Aryl hydrocarbon receptor (AhR)-dependent induction of Jagged 1 (Jag1) expression in AM was necessary and sufficient for the augmentation of allergic airway inflammation by UFP. UFP promoted Th2 and Th17 cell differentiation of allergen-specific T cells in a Jag1- and Notch4-dependent manner. Treatment of mice with an anti-Notch 4 antibody abrogated the exacerbation of allergic airway inflammation induced by UFP.

Conclusion—UFP exacerbate allergic airway inflammation by promoting a Jag1-Notch4-dependent interaction between Alveolar Macrophages and Allergen-Specific T cells, leading to augmented Th cell differentiation.

Graphical Abstract

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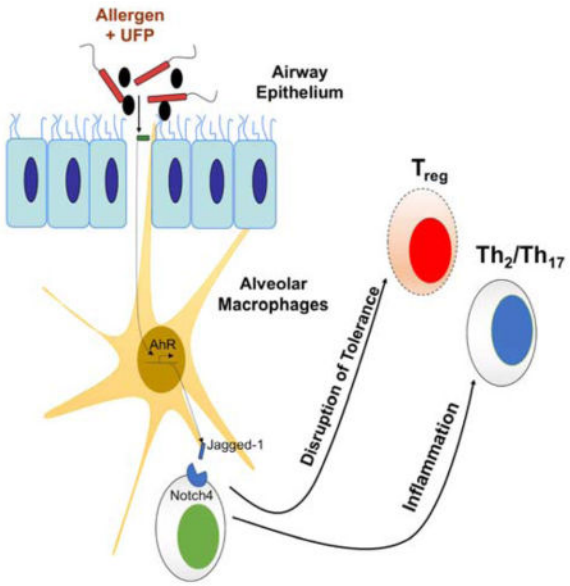

Keywords

Airway hyper-responsiveness; Allergic Airway Inflammation; Alveolar macrophages; Aryl Hydrocarbon Receptor; Asthma; Jagged1; Notch; Notch4; Traffic-Related Particulate Matter; Ultra-Fine Particles

INTRODUCTION

It is well appreciated that exposure to air pollution, especially particulate matter (PM) emitted by combustion sources, play an important role in the increased incidence and prevalence of asthma in recent decades [1–6]. Among air pollutants, exposure to PM shows the strongest correlation with adverse respiratory health effects [7–10]. These particles have been shown to promote Th2 and Th17 cell responses and upregulate IgE production in the exposed host [11–15].

Inhaled PM exhibit differential airway penetrance that stratifies according to size. Unlike coarse particles (CP; ≥2.5 μm in diameter) trapped in the nasopharyngeal region, fine particles (FP; ≤2.5 μm in diameter) and ultra-fine particles (UFP; ≤0.2 μm in diameter) are able to penetrate into the lower respiratory tract where they are taken up by antigen-presenting cells (APC) to mediate local and systemic inflammation [16]. PM modulation of APC function maybe particularly relevant to the adjuvant-like effect of PM in promoting immune responses to allergens [13, 17]. Our recent studies have identified a key mechanism common to both UFP and FP by which they augment allergic responses, involving their induction of the Notch receptor ligand Jagged1 (Jag1) on APC [15]. This induction is mediated by the activation by PM-associated polycyclic aromatic hydrocarbons (PAH) of the aryl hydrocarbon receptor (AhR), which in turn mediates the transcriptional activation of *Jag1*. Jag1 engages Notch receptors on allergen-specific T cells, leading to their augmented differentiation into disease-promoting Th cells. These studies did not precisely identify the

relevant APC species involved in this process, nor the target Notch receptor(s) mediating the response to PM-induced Jag1.

Lung macrophages have previously been implicated in the uptake of and response to PM [18–20]. They include two major subsets: alveolar macrophages (AM), expressing high levels of the β2 integrin CD11c (CD11c^{hi}) and interstitial macrophages (IM) expressing intermediate levels of CD11c (CD11c^{int}) [21, 22]. Studies have shown that both populations promote immune tolerance in the steady state by inducing naive T cell to Treg cell differentiation [23, 24]. However, inflammatory stimuli, including allergens and endotoxin, modulate the expression of co-stimulation molecules and alter the potency of lung macrophage as antigen presenting cells [25, 26]. In a similar vein, exposure of AM to PM alters their function, rendering them pro-inflammatory [27]. In addition to targeting lung macrophages, PM have been shown to potentiate the antigen presenting function of lung dendritic cells (DC) [28]. The relative contribution of the respective APC type to the allergic airway inflammatory response induced by PM remains to be fully elucidated.

To investigate mechanisms by which PM exposure may target lung APC to promote allergic diseases, we employed a range of genetic, immunological and whole animal approaches. Here, we provide evidence for a critical role for PM-mediated, AhR-dependent Jag1 induction in AM in promoting allergic airway inflammation by a process involving Notch4-dependent allergen-specific T helper cell differentiation.

METHODS

Mice

Il4ra^{R576} and *Foxp3*^{EGFP} mice were previously described [15, 29, 30]. The following mice were obtained from the Jax Lab: BLAB/c (WT), *Ahr*$^{fl/fl}$ (Ahr$^{tm3.1Bra}$) [31], *Lyz2*^{Cre} (CreB6.129P2-*Lyz2*$^{tm1(cre)Ifo}$/J) and *CD11c*^{Cre} (B6.Cg-Tg(Itgax-cre)1-1Reiz/J) [32]. *DO11.10Rag2*$^{-/-}$ mice were obtained from Taconic farms. They were crossed with *Il4ra*^{R576} mice to generate *DO11.10Rag2*$^{-/-}$*Il4ra*^{R576}*Foxp3*^{EGFP} mice [30]. *Jag1*$^{fl/fl}$ mice were kindly provided by Dr. Freddy Radtke [33].

Particles

UFP (≤0.18 μm) were collected in an urban area of downtown Los Angeles, as previously reported [15]. Constituent components of the particles were analyzed as described and are summarized in Table E1 in the Online Repository[34]. The respective particles were suspended in an aqueous solution, with the hydrophilic components becoming part of the solution, while the solid non-soluble UFP cores are left in suspension. The entire mixture was administered intranasally, as indicated below

T cell co-cultures with lung macrophages and DC

Naïve CD4^{+}DO11.10^{+} T cells were isolated from spleens of *DO11.10*$^{+}$*RAG2*$^{-/-}$ *Il4ra*^{R576}*Foxp3*^{EGFP} mice by fluorescein-activated cell sorting (FACS). AM and IM were isolated by FACS and were aliquoted at 2×10^{4} cells in 48 well plates, then either sham treated or treated overnight with UFP at 10 μg/ml. The UFP treatment did not induce

increased apoptosis as compared to sham treatment, as assessed by Annexin V staining (data not shown). The APC were washed twice with PBS to remove residual UFP, and the T cells were then added at 4×10^5 cells/well in a final volume of 0.5 ml 10% fetal calf serum/RPMI culture medium. Cultures were treated with the $OVA_{323–339}$ peptide at 1μM, as indicated. Anti-murine Notch Ab were added at 10 μg/ml each, as indicated.

Allergic sensitization and challenge

Mice were sensitized to OVA by intraperitoneal (*i.p.*) injection of 100 μg OVA in100 μl PBS, then boosted two weeks later with a second i.p. injection of OVA in PBS. Control mice were sham sensitized and boosted with PBS alone. Starting on day 29, both OVA and sham-sensitized mice were challenged with aerosolized OVA at 1%, for 30 minutes daily for 3 days. Two hours before each OVA aerosol exposure, subgroups of mice were given intransally (*i.n.*) either PBS or UFP at 10 μg/100μl PBS/instillation. For anti-Notch4 antibody blocking, 150 μg Armenian hamster anti-mouse Notch4 IgG mAb (clone HMN4-14; Bio X Cell) [35], or control Armenian hamster IgG polyclonal antibodies (Ab) (Bio X cell), were suspended in 100 μl PBS buffer and administered daily for three consecutive days during OVA aerosol challenge. Mice were euthanized on day 32 post sensitization and analyzed. For dust mite-induced allergic airway inflammation, mice received 5μg of lyophilized *D. Pteronyssinus* extract (Greer) in 100 μl PBS intranasally for 3 days at the start of the protocol then challenged with the same dose of *D. Pteronyssinus* extract on days 15–17 with or without UFP. Mice were euthanized on day 18 and analyzed for measures of airway inflammation. Bronchoalveolar lavage (BAL) fluid and lung tissues was obtained and analyzed for cellular components and T cell cytokine expression following previously published methods [15, 30].

For AM, IM and DC cell transfer studies, cells were isolated from either $Il4ra^{R576}$ or $Il4ra^{R576}Lyz2^{Cre}Jag1^{\Delta/\Delta}$ donor mice by FACS. The cells were cultured overnight and either sham treated, or loaded with $OVA_{323\text{-}339}$ peptide at 5 nM peptide concentration either alone or together with UFP at 10 μg/ml. The cells were transferred intratracheally to OVA-sensitized $Il4ra^{R576}Lyz2^{Cre}Jag1^{\Delta/\Delta}$ recipient mice at 10^5 cells/mouse repeated twice over two days. The mice were euthanized on the third day and analyzed for the different parameters of allergic airway inflammation.

Lung histopathology staining

Paraffin-embedded lung sections were stained with hemtoxylin and eosin (H&E) as described [36]. The lung pathology was scored by blinded operators. Inflammation was scored separately for cellular infiltration around blood vessels and airways: 0, no infiltrates; 1, few inflammatory cells; 2, a ring of inflammatory cells 1 cell layer deep; 3, a ring of inflammatory cells 2–4 cells deep; 4, a ring of inflammatory cells of >4 cells deep [37]. A composite score was determined by the adding the inflammatory scores for both vessels and airways. The number and distribution of goblet cells was assessed by Periodic Acid Schiff (PAS) staining of mucin granules. Individual airways (bronchi/bronchioles) were scored for goblet cell hyperplasia according to the following scale: 0, no PAS-positive cells; 1, <5% PAS-positive cells; 2, 5 to 10% PAS-positive cells; 3, 10 to 25% PAS-positive cells; and 4, > 25% PAS-positive cells[38].

Statistical analysis

Student's two-tailed *t*-test, one and two way ANOVA and repeat measures two way ANOVA with Bonferroni post-test analysis of groups were used to compare test groups, as indicated. A p value <0.05 was considered statistically significant.

Study approval

All animal studies were reviewed and approved by the Boston Children's Hospital office of Animal Care Resources.

Other Methods

Information real time PCR analysis, flow cytometry (including Fluoresbrite® yellow green (YG) microspheres and nanobeads), intracellular staining reagents, Ab and methods, IgE ELISA and measurement of airway hyper-responsiveness are provided in the Methods section in this article's Online Repository at www.jacionline.org.

RESULTS

Lung Macrophages are the major cellular target of UFP in the lungs under basal and inflammatory conditions

To further elucidate the role of the Jag1-AhR-Notch pathway in the promotion of airway inflammation by UFP, we first sought to establish detailed immunophenotypic characterization of the APC targeted by PM in our study model, both under basal and especially allergic inflammatory conditions. We focused in our studies on UFP, which are particularly toxic by virtue of their deep penetrance, large surface area to size ratios, higher content per mass of PAH and greater capacity to induce oxidative stress [39, 40]. We employed an OVA-induced allergic airway inflammation model using the *Il4ra*^{R576} mice. These mice carry an IL-4 receptor alpha chain (IL-4Rα-R576) variant that mediates exaggerated allergic airway inflammation to allergen, alone or in combination with UFP, by virtue of mediating IL-4R-dependent mixed Th2/Th17 cell inflammation [15, 30]. Mice were sensitized by intra-peritoneal injection of OVA and then challenged by repeated inhalation of PBS (sham challenge) or 1% aerosolized OVA. Subgroups of mice were treated intra-nasally with UFP in combination with Fluoresbrite® YG nanobeads (0.05 μm effective diameter) or microspheres (1 μm effective diameter) 2hr before the OVA aerosol challenge [41]. Nanobead fluorescence positive cell populations were analyzed by flow cytometry (Fig 1, *A* and *B*). In the absence of inflammation (PBS-sensitized, OVA-treated group), ≥95% nanobead-uptaking cells were CD45^{+}F4/80^{+}CD64^{+}MHCII^{+}CD11b^{Int}CD11c^{Hi} alveolar macrophage whereas CD45^{+}F4/80^{+}CD64^{+}MHCII^{+}CD11b^{Hi}CD11c^{Int} interstitial macrophage represented 2–3% of total microsphere positive cells (Fig E1–E3 in the Online Repository) [42]. In the context of allergic airway inflammation induced by OVA and especially by OVA+UFP, about 85% of nanobeads-uptaking cells were macrophages, distributed at a 70:30 ratio between AM and IM (Fig 1 *A* –C). The particle-uptaking AM population was CD38^{Int}Egr2^{Hi} (M2-like), while the IM population was CD38^{Hi}Egr2^{Int} (M1-like) (Fig E3, *A* and *B* in the Online Repository) [43]. In agreement with their M2-like phenotype, *in vitro* treatment of cell-sorted AM with UFP sharply upregulated their production of the cytokines IL-10, CCL17, IL-6 and

TNF-α but induced little change in their baseline production of IL-12 (Fig E3, *C*) [44, 45]. Of the remaining beads (15%), about 2/3rd were picked up by $CD11c^{hi}CD11b^{int}CD8\alpha^{+}CD103^{+}B220^{-}PDCA1^{-}$ classical (c)DCs [46], and the rest by $Gr1^{+}SiglecF^{low}$ neutrophils (Fig 1, *B* and C, and Fig. E1 in the Online Repository). These results indicated that alveolar and interstitial macrophage were the major cell subset responsible for the clearance of inhaled UFP in the context of allergic airway inflammation. Similar cellular localization results were obtained when Fluoresbrite® YG microsphere beads were used instead of nanobeads, consistent with our previously published data showing FP and UFP sharing the same AhR-Notch-Jag1 mechanism of action in promoting allergic airway inflammation (Fig. E4 in the Online Repository).

UFP differentially induce Jag1 expression in lung AM

UFP induces Jag1 expression in an AhR-dependent manner in bone marrow-derived dendritic cells [15] and in macrophages (Fig E5, *A* in the Online Repository). We examined the expression of *Jag1* transcripts in different APC populations isolated from the lungs of $Il4ra^{R576}$ mice and either sham treated or treated *in vitro* with UFP. Jag1 expression was highest at baseline in AM as compared to IM and DC (Fig 1, *D*). *In vitro* treatment with UFP super-induced *Jag1* transcript expression in AM, whereas the same treatment was associated with modest increases in IM and DC (Fig 1, *D*). In contrast, treatment with Fluoresbrite® YG nanobeads failed to induce Jag1 expression on AM (Fig E2, *A*). It also failed to affect airway inflammation induced by OVA despite the Fluoresbrite® YG nanobeads localizing to AM when administered intranasally (Fig E2, *B*– *D*). Deletion of a floxed *Ahr* allele by means of a Cre recombinase driven by the lysozyme 2 gene promoter ($Lyz2^{Cre}$), which is active in myeloid-lineage cells, greatly reduced baseline expression of *Jag1* transcripts in AM of $Il4ra^{R576}Lyz2^{Cre}Ahr^{\Delta/\Delta}$ mice and abolished its super-induction by UFP. While a similar trend was also noted in the other cell types, there was partial sparing of *Jag1* expression in DC. Flow cytometric analysis confirmed the heightened expression of Jag1 in AM as compared to the other cell types and its downregulation upon *Ahr* deletion (Fig 1, *E*). UFP-induced Jag1 expression in bone marrow-derived macrophages was similarly affected by $Lyz2^{Cre}$-driven *Ahr* deletion (Fig E5 in the Online Repository). Furthermore, sensitization of mice with OVA followed by challenge with OVA and UFP resulted in the preferential induction of Jag1 on AM as compared to IM and DC, and this induction was reversed upon by $Lyz2^{Cre}$-driven *Ahr* deletion (Fig E5, *B*).

These results were further ascertained by the deletion of a floxed *Jag1* gene in myeloid lineages using $Lyz2^{Cre}$ ($Il4ra^{R576}Lyz2^{Cre}Jag1^{\Delta/\Delta}$) (Fig 1, *F and G*). *Jag1* transcript expression and Jag1 surface staining were completely abrogated in AM both at baseline and following UFP treatment. Reduced *Jag1* transcript expression and Jag1 protein staining persisted in IM, while their levels were unaffected in DC. These findings confirmed AM as the principal APC cell type expressing Jag1 both at baseline and following UFP treatment and that this expression proceeds by an *Ahr*-dependent mechanism. They also showed that $Lyz2^{Cre}$ preferentially targets *Jag1* in macrophages, particularly AM, while largely sparing it in DC, consistent with previous lineage tracing analysis on the activity of $Lyz2^{Cre}$ in macrophages versus dendritic cells [47].

UFP-treated AM promote Th cell differentiation in a Jag1-dependent mechanism

To examine if induction of Jag1 expression in AM by UFP augments allergen-induced Th cell differentiation, we employed an *in vitro* Th cell differentiation system involving naïve *Il4ra*^{R576}DO11.10^{+}CD4^{+} T cells. Naïve DO11.10^{+}CD4^{+} T cells were incubated with FACS-purified AM isolated from *Il4R*^{576} or *Il4ra*^{R576}*Lyz2*^{Cre}*Jag1*$^{\Delta/\Delta}$ mice. The AM were either sham pulsed with PBS or pulsed with the OVA peptide OVA$_{323-339}$, alone or together with UFP. At the end of the incubation period, Th cell cytokine expression was analyzed in gated CD4^{+}Foxp3^{-} (non-regulatory) T cells. Co-culture with OVA$_{323-339}$ peptide-pulsed *IL4R*^{R576} AM resulted in increased production by DO11.10^{+}CD4^{+}Foxp3^{-} T cells of IL-17, IL-13 and IL-4, and to much lesser extent IFN-γ, as revealed by flow cytometric staining (Fig E6 in the Online Repository and Fig 2). Expression of the first three cytokines was markedly upregulated by the addition of UFP (10 μg/ml), whereas that of IFN-γ was down-regulated, consistent with exaggerated Th2/Th17 skewing [30]. In contrast, the induction of IL-17, IL-13 and IL-4 expression in DO11.10^{+}CD4^{+} T cells by OVA$_{323-339}$ was moderately inhibited, and their super-induction by UFP completely abolished, when *Il4ra*^{R576}*Lyz2*^{Cre}*Jag1*$^{\Delta/\Delta}$ AM were used as APC. IFN-γ expression in those co-cultures was also profoundly impaired (Fig 2, *A* and *B*).

We also employed the DO11.10 cell *in vitro* Th cell differentiation system to examine the impact of UFP treatment on the capacity of AM to support the differentiation of naïve allergen-specific T cells into induced Treg cells. In the absence of UFP, OVA$_{323-339}$-loaded AM drove the differentiation of up to 40% of naïve *Il4ra*^{R576}DO11.10^{+}CD4^{+} T cells into Foxp3^{+} induced T regulatory (iTreg) cells. Treatment with UFP partially inhibited iTreg cell differentiation independent of Jag1 expression (Fig 3, *A* and *B*). Critically however, UFP treatment of OVA$_{323-339}$ peptide-presenting AM skewed the formed iTreg cells into secreting Th2/17 cell cytokines, including IL-4, IL-13 and IL-17, but not the Th1 cytokine IFN-γ. This skewing was largely reversed by deletion of *Jag1* in AM (Fig 3, *C* and *D*). These results indicated that UFP adversely affected allergen-specific iTreg cell differentiation, in part by destabilizing newly formed iTreg cells towards Th2/17 cell differentiation in a Jag1-dependent manner.

Jag1 deletion in myeloid lineages abolishes the augmentation of allergic airway inflammation by UFP

To determine the role of Jag1 expression on AM in supporting UFP upregulation of allergic airway inflammation *in vivo*, we first employed *Lyz2*^{Cre} to delete component genes of the *Ahr-Jag1* genetic circuit in myeloid lineage cells. Accordingly, *Il4ra*^{R576}*Lyz2*^{Cre}*Jag1*$^{\Delta/\Delta}$ mice and *Il4ra*^{R576} mice were sensitized by intra-peritoneal injection of OVA and then challenged by inhalation of 1% aerosolized OVA. Control mice were sham sensitized with PBS and challenged with aerosolized OVA. Subgroups of mice were treated intra-nasally with UFP (10 μg/instillation) or PBS 2 hr before the OVA aerosol challenge [41]. Sensitization and challenge of *Il4ra*^{R576} mice with OVA resulted in a robust airway inflammatory response, characterized by airway inflammation and hyper-responsiveness, eosinophilia and T cell infiltration in the BAL fluid, elevated total and OVA-specific serum IgE responses, and augmented Th2 and Th17 cell responses (Fig. 4A–K). It additionally induced Treg cell destabilization into Th cell like phenotypes (Fig 4, L–O). All these parameters were

markedly augmented by UFP exposure during the OVA challenge phase. OVA sensitization and challenge of *Il4ra*^{R576}*Lyz2*^{Cre}*Jag1*$^{\Delta/\Delta}$ mice also resulted in a robust allergic airway inflammatory response that was similar to that noted in OVA sensitized and challenged *Il4ra*^{R576} mice. However, the augmentation by UFP of all the aforementioned parameters of allergic airway inflammation was completely abrogated in *Il4ra*^{R576}*Lyz2*^{Cre}*Jag1*$^{\Delta/\Delta}$ mice, indicating a requisite requirement for Jag1 expression in myeloid lineages for UFP to exert its pro-inflammatory effects in the airways (Fig. 4A–O).

We also examined the role of Jag1 expression in myeloid lineage cells to mediate the augmentation by UFP of allergic airway inflammation induced upon the intranasal treatment of *Il4ra*^{R576} mice with extracts of house mites (*D. pteronyssinus*), a common and potent human allergen. Results were concordant with those observed with OVA sensitization and challenge. Whereas UFP augmented the airway inflammation induced by treatment of *Il4ra*^{R576} mice with a low dose of *D. pteronyssinus* (5 μg), this effect was abrogated in *Il4ra*^{R576}*Lyz2*^{Cre}*Jag1*$^{\Delta/\Delta}$ mice (Fig. E7 in the Online Repository). Myeloid lineage-specific deletion of *Ahr* in *Il4ra*^{R576} mice (*Il4ra*^{R576}*Lyz2*^{Cre}*Ahr*$^{\Delta/\Delta}$) also abrogated the capacity of UFP to augment the various parameters of allergic airway inflammation induced by OVA, consistent with the requirement for AhR signaling for the induction of Jag1 expression by UFP (Fig E8 in the Online Repository). In contrast, deletion of *Jag1* in all CD11c^{+} APC lineages using a *CD11c*^{Cre} did not inhibit the promotion of airway inflammation by UFP and in fact worsened it, indicating a unique and specific requirement for Jag1 induction by UFP in AM for their acquisition of a pro-inflammatory function (Fig E9 in the Online Repository).

Jag1 expression in AM is sufficient to mediate UFP upregulation of allergic airway inflammation

To specifically establish the role of Jag1 expression in AM in the exacerbation of allergen-induced airway inflammation by UFP, we examined the capacity of AM to rescue the UFP effect when transferred into *Il4ra*^{R576}*Lyz2*^{Cre}*Jag1*$^{\Delta/\Delta}$ mice. Accordingly, AM were isolated from either *Il4ra*^{R576} or *Il4ra*^{R576}*Lyz2*^{Cre}*Jag1*$^{\Delta/\Delta}$ mice, either sham treated or loaded with OVA$_{323\text{-}339}$ peptide in the absence or presence of UFP. The cells were transferred into the airways of *Il4ra*^{R576}*Lyz2*^{Cre}*Jag1*$^{\Delta/\Delta}$ mice that were sensitized with OVA, which were then examined for induction of allergic airway inflammation. Results revealed that transfer of Jag1-sufficient OVA$_{323\text{-}339}$ peptide-pulsed and UFP-treated *Il4ra*^{R576} AM into OVA-sensitized *Il4ra*^{R576}*Lyz2*^{Cre}*Jag1*$^{\Delta/\Delta}$ mice recapitulated all the stigmata of exacerbated allergic airway inflammation induced by UFP, including augmented tissue inflammation, increased airway hyper-responsiveness, serum total and OVA-specific IgE, and BAL fluid CD4^{+} T cell infiltration and eosinophilia (Fig 5, *A–G*). It also augmented Th cell cytokine production and Treg cell destabilization into Th cell like phenotypes (Fig 5, *H–O*). In contrast, transfer of similarly treated Jag1-deficient *Il4ra*^{R576}*Lyz2*^{Cre}*Jag1*$^{\Delta/\Delta}$ AM failed to do so, indicating that Jag1 expression of AM is sufficient to restore the capacity of UFP to augment allergen-induced airway inflammation.

We also examined the capacity of IM and DC isolated from the lungs of *Il4ra*^{R576} or *Il4ra*^{R576}*Lyz2*^{Cre}*Jag1*$^{\Delta/\Delta}$ mice and sham treated or loaded with OVA$_{323\text{-}339}$ or OVA$_{323\text{-}339}$ +

UFP to promote airway inflammation when transferred intra-tracheally into OVA-sensitized *Il4ra*^{R576}*Lyz2*^{Cre}*Jag1*$^{\Delta/\Delta}$ mice. However, unlike the case of Jag1-sufficient AM, transferred IM failed to promote allergic airway inflammation irrespective of their treatment modality (Fig E10, *A–F* in the Online Repository). The transferred DC also failed to induced airway hyper-responsiveness. $OVA_{323-339}$ –loaded DC induced suboptimal tissue infiltration with eosinophils and Th cells as compared to AM, which was not augmented by UFP treatment. These results are consistent with the unique role of Jag1-sufficient AM in promoting airway inflammation and in its super-induction by UFP (Fig E10, *G–L* in the Online Repository).

Promotion by UFP of allergen-induced Th cell differentiation involves Jag1-Notch4 interaction

We next determined the role of the respective Notch receptors in mediating the pro-inflammatory effects of Jag1 expressed by AM upon UFP treatment. Accordingly, we first analyzed the ex-vivo expression of the different Notch receptors in FACS-purified $CD4^+EGFP^-$ T conventional cells and $CD4^+EGFP^+$ Treg cells, isolated from *Il4ra*^{R576}Foxp3^{EGFP} mice. The cells were isolated from the spleens of unmanipulated mice and from the lungs of mice subjected to allergic airway inflammation without or with UFP co-treatment. Splenic $CD4^+$ T cells primarily expressed Notch1 and Notch2 (data not shown), consistent with our previous results [48]. In contrast, Notch4 expression was upregulated in $CD4^+$ T cells isolated from the lungs of mice sensitized with OVA and challenged with OVA+UFP, to become the highest among the four Notch receptors (Fig E11 in the Online Repository). Notch1 and Notch2 expression was also upregulated but to a lesser extent, while that of Notch3 decreased.

Informed by the above results, we next employed the *in vitro* co-culture system described in Fig 2 to determined the capacity of neutralizing Notch1, 2 and 4 Ab to reverse the augmentation by UFP treatment of $OVA_{323-339}$ peptide-presenting AM of Th cell cytokine production by responding DO11.10 T cells. FACS-purified Jag1-sufficient (*Il4ra*^{R576}) or -deficient (*Il4ra*^{R576}*Lyz2*^{Cre}*Jag1*$^{\Delta/\Delta}$) AM were either sham treated or treated with $OVA_{323-339}$ peptide, either alone or together with UFP. They were co-cultured with naïve *Il4ra*^{R576}$DO11.10^+CD4^+$ T cells in the presence of either isotype control mAbs or neutralizing mAbs specific for individual Notch receptors, and the T cells were examined for Th cell cytokine expression. As expected, UFP treatment of $OVA_{323-339}$ peptide-pulsed *Il4ra*^{R576} AM upregulated the production by $DO11.10^+CD4^+Foxp3^-$ T cells of IL-17, IL-13 and IL-4, and to much lesser extent IFN-γ, whereas this effect was abolished when *Il4ra*^{R576}*Lyz2*^{Cre}*Jag1*$^{\Delta/\Delta}$ AM were used as APC. Critically, upregulation of Th cell cytokine expression by UFP, including IL-4, IL-13 and IL-17, was uniformly inhibited by co-treatment with a highly specific neutralizing anti-Notch4 mAb (Fig 6 and Fig E12 in the Online Repository) [49]. Anti-Notch4 mAb also inhibited the residual IFN-γ production induced by OVA, alone or with UFP. In contrast, treatment with neutralizing mAbs specific for other Notch receptors gave partial and/or selective inhibitory results (Fig E12 in the Online Repository). Anti-Notch4 mAb also suppressed the production by $DO11.10^+CD4^+Foxp3^+$ iTreg cells of Th cell cytokines when cultured with $OVA_{323-339}$ peptide-presenting AM that were treated with UFP (Fig E13 in the Online Repository). Of note, treatment with the anti-Notch4 occasionally suppressed residual Th cell cytokine

production (e.g. IL-4 production) beyond what could be accounted for by Jag1 activation, suggesting an additional contribution by other Notch ligands acting via Notch4 in supporting those Th cell responses.

The specificity and efficacy of the anti-Notch4 mAb in blocking Notch signaling in allergen-specific T cells was further ascertained in *in vitro* co-cultures of $OVA_{323-339}$ and UFP-treated AM with *Il4ra*^{R576}CD4^{+}DO11.10^{+} T cells, in which treatment with anti-Notch4 mAb blocked the transcriptional upregulation of the Notch target genes *Hes1*, *Hey1* and *Nrarp* (Fig E14 in the Online Repository). Overall, these findings implicated Notch4 as a key Notch receptor through which Jag1 mediates the inflammatory responses to UFP by promoting Th cell differentiation.

Notch4 inhibition suppresses the exacerbation of allergic airway inflammation by UFP

Given the efficacy of the neutralizing anti-Notch4 mAb in reversing the augmented *in vitro* differentiation of allergen-specific Th cells induced by UFP treatment of allergen peptide-presenting AM, we examined the impact of inhibiting Notch4 on the exacerbation of the allergic airway inflammatory response induced by UFP. Accordingly, *Il4ra*^{R576} mice sensitized and challenged with OVA alone or together with UFP, were treated with either an anti-Notch4 or an isotype control mAb during the challenge phase then analyzed for the various parameters of the airway allergic inflammatory response. *In vivo*, treatment with the anti-Notch4 mAb had little or no effect on OVA-induced allergic airway inflammation in terms of tissue inflammation, airway hyper-responsiveness, BAL fluid eosinophilia, serum total and OVA-specific IgE response, and airway Th2 and Th17 cell responses. In contrast, it completely inhibited the potentiation of the aforementioned parameters induced by UFP, thus implicating Notch4 in mediating the potentiating effects of UFP on allergic airway inflammation (Fig 7, A–P).

DISCUSSION

Our previous studies have demonstrated that traffic-related PM, including UFP and FP, promotes allergic airway inflammation by inducing Jag1 expression on APCs in an AhR-dependent manner, which in turn activates Notch signaling to augment Th cytokine expression by allergen-specific T cells [15]. In this study, we have identified AM as the key target of PM by virtue of their avid uptake of nano and micro particles and their superinduction of Jag1 expression upon PM uptake as compared to other lung APCs. We have also identified Notch4 on T cells as a key mediator of the Jag1-dependent upregulation by UFP-treated AM of allergen-specific Th cell differentiation and iTreg cell destabilization. Notch4 inhibition by means of a neutralizing anti-Notch4 mAb completely abrogated the upregulation by UFP of allergic airway inflammation. These studies thus established cellular elements, including AM and allergen-specific CD4^{+} Th and Treg cells, and molecular effectors, including Jag1 and Notch4, involved in mediating the pro-inflammatory effects of the PM-activated AhR-Jag1-Notch circuit in allergic airway inflammation.

AM have been implicated in the homeostatic maintenance of tolerance in the airways by virtue of their down regulation of the antigen presenting capacity of DC [50], as well as their promotion of iTreg cell differentiation [23]. AM are also less effective in presenting antigens

as compared to DC, a defect that could be overcome by the provision of an accessory signal such as co-stimulation of T cells with CD28 or IL-2 [51, 52]. Upregulation of Jag1 expression in AM by PM-mediated activation of AhR may enable efficient antigen presentation with Jag1-Notch acting as a co-receptor pair that amplifies Th cell cytokine production [53]. Our results clearly demonstrate a necessary and sufficient role for Jag1-sufficient AM to rescue the augmentation by UFP of allergic airway inflammation in mice lacking Jag1 in their myeloid lineages. Under inflammatory conditions, increased uptake of nanoparticles by IM was noted, possibly reflecting in part the increased abundance of the latter cells in inflamed lung tissues and/or their heightened avidity for these particles. Nevertheless, reconstitution of *Il4ra*^{R576}*Lyz2*^{Cre}*Jag1*$^{\Delta/\Delta}$ mice with either IM or DC failed to rescue the inflammatory responses to UFP, indicative of the requisite role of Jag1-sufficient AM in this process.

While deletion of Jag1 on AM reversed UFP-induced augmentation of allergic airway inflammation, it also attenuated a few parameters of allergen-induced airway inflammation in the absence of UFP, such as the mobilization of $CD4^+$ T cell in lung tissues (Fig 4 and 5). These findings argue that in the absence of UFP treatment residual Jag1 expression on AM may contributes to allergen-induced airway inflammation, and that UFP acts to greatly amplify this process.

A surprising finding in our studies was the identification of Notch4 as a key Notch receptor through which UFP-mediated their effects in upregulating allergic airway inflammation. Notch4 inhibition provided effective and uniform suppression of UFP and AM-dependent *in vitro* differentiation of allergen-specific T cells into different Th cell subsets. In contrast, inhibition of other Notch receptors, including, including Notch1 and Notch2, provided selective and/or partial inhibition of Th cell cytokine expression. Notch4 inhibition also suppressed the exacerbation by UFP of allergic airway inflammation in mice. The *NOTCH4* locus has previously been associated with severe asthma [54], suggesting that this pathway may modulate disease severity, especially in as it relates to environmental exposures such as to UFP.

The mechanisms by which Notch4 may selectively contribute to allergic airway inflammation are currently unclear. Jag1 expressed on AM may preferentially interact with Notch4 as compared to other Notch receptors. Alternatively or in parallel, Notch4 may act to differentially amplify the production of Th cell cytokines, or possibly instruct their specific production, as compared to other Notch receptors by means of Notch canonical and non-canonical signaling mechanisms [53, 55]. Notch4 signaling also destabilized differentiating of iTreg cells, leading to their production of Th cell cytokines. Such an iTreg cell phenotype is associated with decreased suppressive function and lineage instability, potentially leading to the terminal differentiation of Treg cells into Th cell lineages [30, 56]. Our previous studies have documented an inhibitory function of Notch signaling in Treg cells in controlling their regulatory functions [48]. The differential contribution of Jag1-Notch4 signaling in allergen-specific Th versus iTreg cells in mediating disease exacerbation by UFP remains to be determined. Also unclear is whether Notch4 signaling in Treg cells plays a unique, non-redundant role in regulating their function as compared to other Notch receptors. Distinct, dedicated functions of different Notch receptors in Th and Treg cell populations in allergic airway inflammation may offer opportunities for targeted therapeutic interventions. Finally,

and in addition to targeting T cells, a neutralizing Notch4 mAb may act *in vivo* to modulate additional cellular elements, such as the vascular endothelium, involved in mobilizing the airway inflammatory response [57, 58]. Future studies will be required to delineate the respective contributions of these mechanisms to UFP-promoted allergic airway inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Ab	Antibodies
AhR	Aryl hydrocarbon receptor
APC	Antigen presenting cells
BAL	Bronchoalveolar lavage
FACS	Fluorescein-activated cell sorting
FP	Fine particles
Jag1	Jagged 1
RL	Lung resistance
PM	Particulate matter
UFP	Ultrafine particles
YG	Yellow green

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Key messages

- Lung alveolar macrophages (AM) are a key target of traffic related particulate matter (PM) in promoting allergic airway inflammation.
- Traffic-related ultra fine particles (UFP) induce the expression of the Notch ligand Jagged 1 (Jag1) on AMs via aryl hydrocarbon receptor
- Jag1 on AM interacts with Notch4 on naïve allergen-specific T cells to promote Th2 and Th17 cell differentiation
- Treatment with an anti-Notch 4 mAb abrogates the exacerbation of allergic airway inflammation induced by UFP.

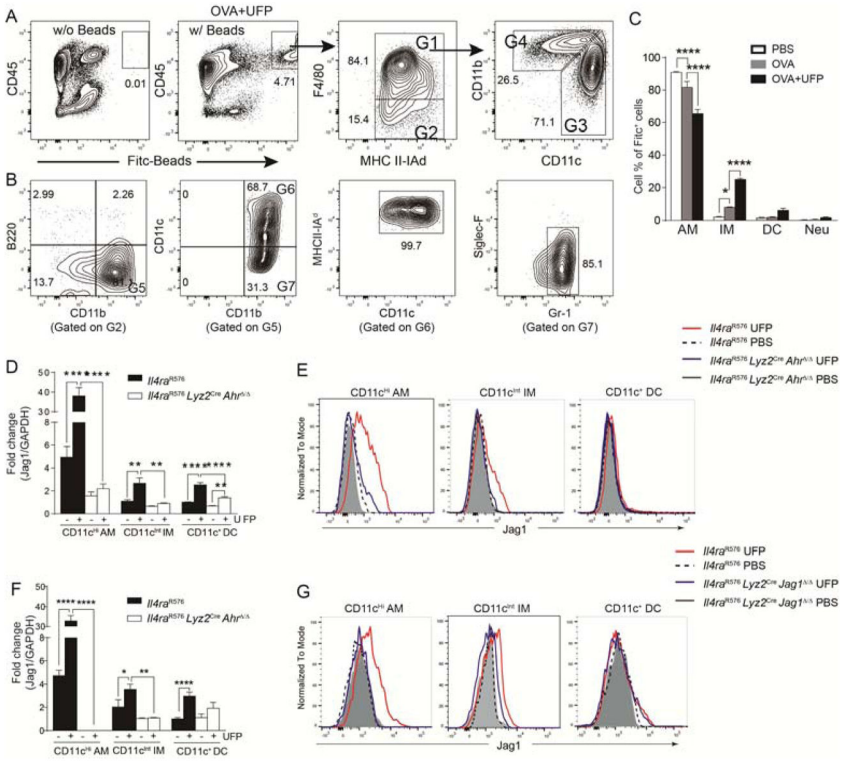

Fig. 1.

AM differentially uptake nanoparticles and highly express Jag1 in response to UFP. **A** and **B,** Flow cytometric analysis of the uptake of fluorescent nanoparticles by different lung cell populations in mice subjected to OVA+UFP-induced allergic airway inflammation. **C,** Bar graph presentation of the distribution of nanoparticles among lung macrophages (AM and IM), dendritic cells (DC) and neutrophils (Neu). **D and E**, Relative fold changes in *Jag1* transcripts, quantitated by RT-PCR (Fig 1, *D*), and flow cytometric analysis of Jag1 expression (Fig 1, *E*), in lung APCs purified from either *Il4ra*^{R576} or *Il4ra*^{R576}*Lyz2*^{Cre}*Ahr*$^{\Delta/\Delta}$ mice and treated with PBS or UFP (10 μg/ml). **F and G**, Relative fold changes in *Jag1* transcripts (Fig 1, *F*), and Jag1 expression (Fig 1, *G*), in lung APCs purified from either *I4ra*^{R576} or *Il4ra*^{R576}*Lyz2*^{Cre}*Jag1*$^{\Delta/\Delta}$ mice and treated with PBS or UFP, as described for Fig 1, *D* and *E*. Results are representative of 2 independent experiments. N=3 mice/group. *p<.05; **p<.01; ***p<.001, ***p<.0001 by one-way ANOVA with post-test analysis (panels *C*, *D* and *F*) or Student's unpaired two-tailed *t* test (panel *F*, CD11c^{+} DC group comparison).

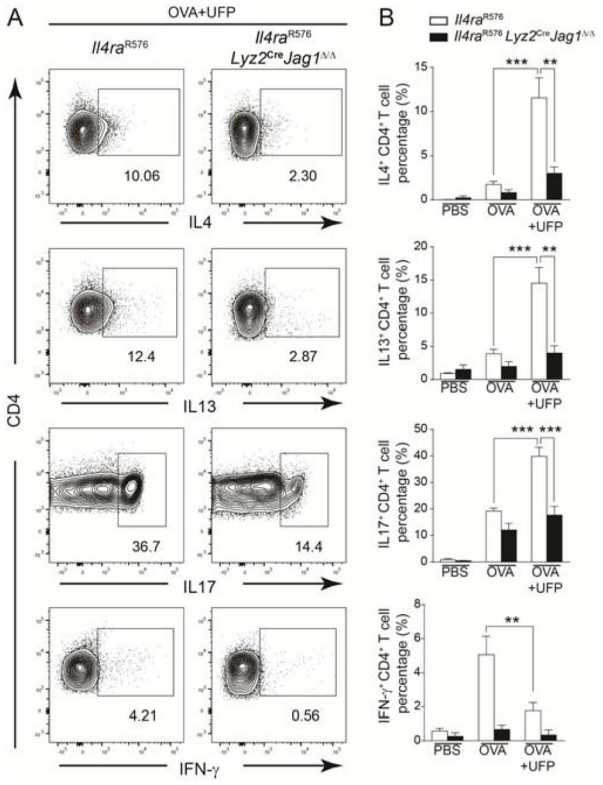

Fig. 2.

AM support UFP-dependent upregulation of Th cell cytokine production by allergen-specific $CD4^+$ T cells in a Jag1-dependent manner. **A**, Representative flow cytometric analysis of IL-4, IL-13, IL-17 and IFN-γ cytokine production by naive *Il4ra*^{R576}$CD4^+$$DO11.10^+$ T cells co-cultured with FACS-purified AM isolated from *l4ra*^{R576} or *Il4ra*^{R576}*Lyz2*^{Cre}*Jag1*$^{Δ/Δ}$ mice pulsed with $OVA_{323-339}$ peptide in the presence of UFP (10 μg/mL). Cytokine expression was analyzed in gated $CD4^+Foxp3^-$ T cell. **B**, Frequencies of $CD4^+Foxp3^-$ T cells expressing the respective cytokine upon co-culture with AM that have been either sham treated (PBS) or pulsed with $OVA_{323-339}$ peptide, alone or in the presence of UFP. Results are representative of 3 independent experiments. *P < .05, **P < .01, ***P < .001, and ****P < .0001, two-way ANOVA with post-test analysis.

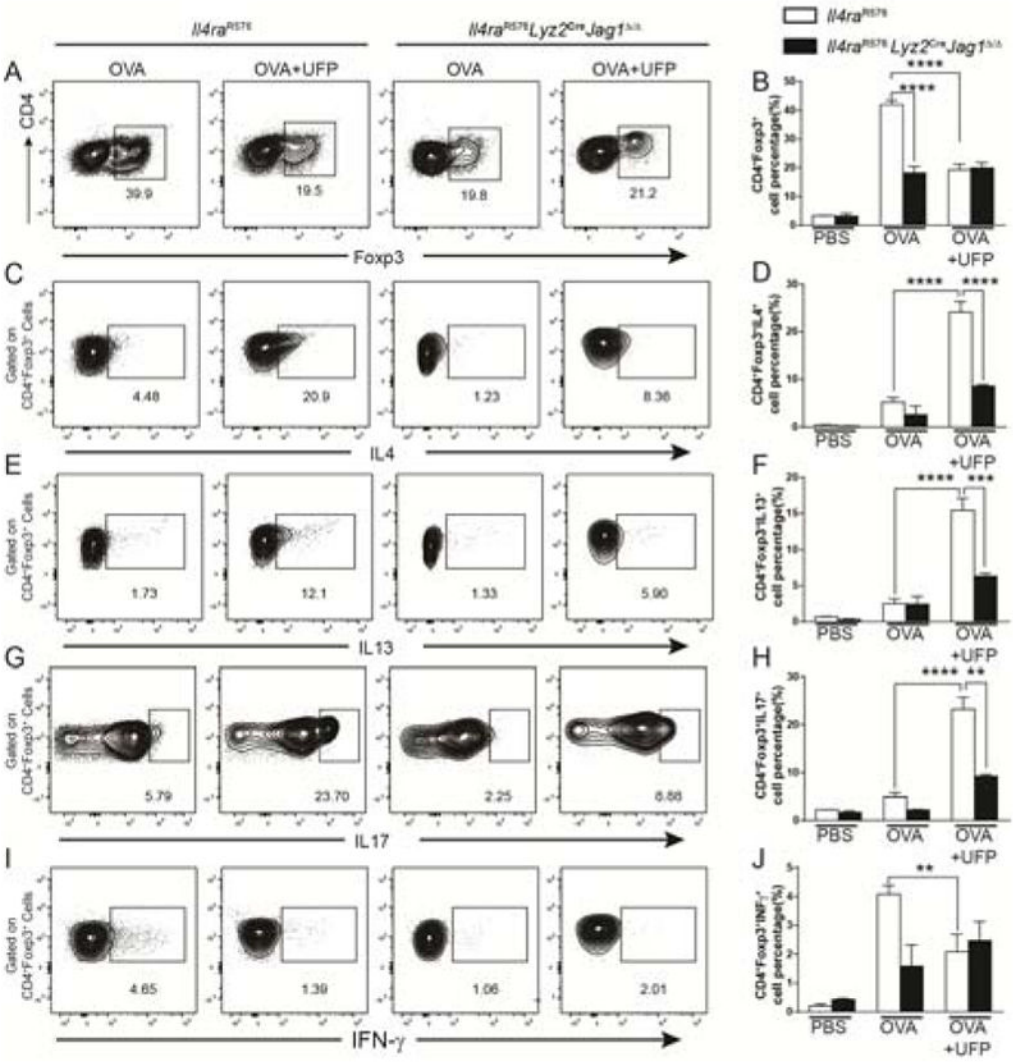

Fig. 3.

UFP skews AM-dependent iTreg cell differentiation towards Th2/17 cell like phenotypes in Jag1-dependent manner. Representative flow cytometric analysis and frequencies of $CD4^+Foxp3^+$ iTreg cells (Fig 3, *A* and *B*), and their expression of IL-4, IL-13, IL-17, and IFN-g (Fig 3, *C-J*), on co-culture of naive $Il4ra^{R576}CD4^+DO11.10^+$ T cells with FACS-purified AM isolated from $Il4ra^{R576}$, or $Il4ra^{R576}Lyz2^{Cre}Jag1^{\Delta/\Delta}$ mice. The AM that have been either sham treated (PBS) or pulsed with OVA323-339 peptide either alone or in the presence of UFP, as indicated. Results are representative of 3 independent experiments. **P < .01, ***P < .001, and ****P < .0001, two-way ANOVA with post-test analysis.

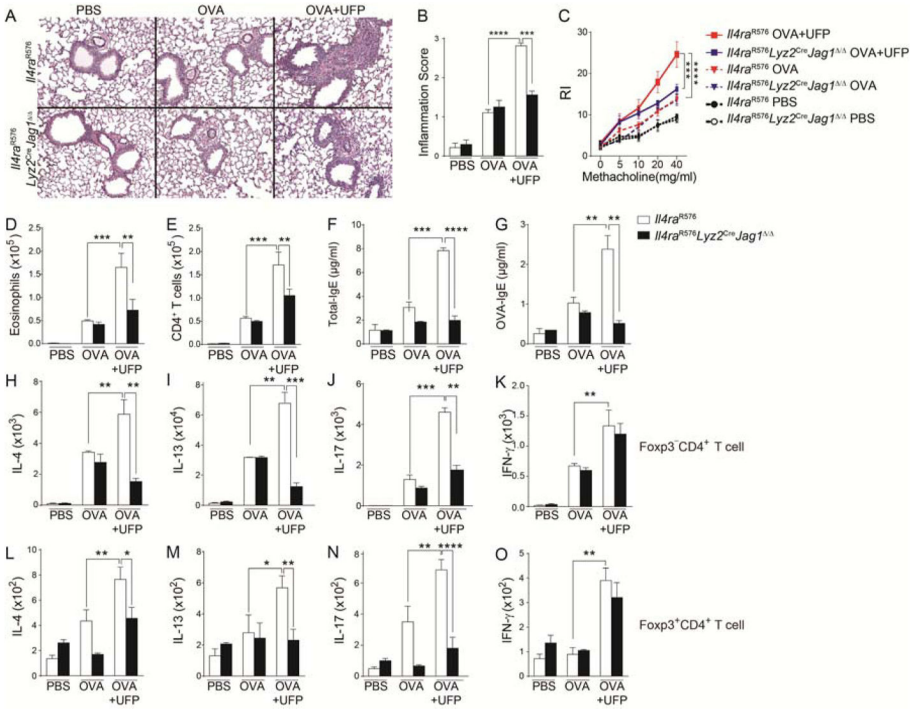

Fig. 4.

Myeloid lineage-specific deletion of *Jag*1 confers protection against UFP-induced exacerbation of allergic airway inflammation. **A,** Representative PAS-stained sections of lung isolated from *Il4ra*^{R576} or *Il4ra*^{R576}*Lyz2*^{Cre}*Jag1*$^{\Delta/\Delta}$ mice in PBS, OVA or OVA+UFP groups (200X magnification). **B,** Inflammation scores in the lung tissues isolated from the mouse groups described in Fig 4, *A*. **C–G** Airway hyper-responsiveness in response to methacholine (Fig 4, *C*), absolute numbers of eosinophils (Fig 4, *D*) and T cells (Fig 4, *E*) in the BAL fluids, total (Fig 4, *F*) and OVA-specific (Fig 4, *G*) levels in the serum of the mouse groups described in Fig 4, *A*. **H–K,** Absolute numbers of lung Foxp3^{-}CD4^{+}T cells secreting IL-4 (Fig 4, *H*), IL13 (Fig 4, *I*), IL-17 (Fig 3, *J*) and IFN-γ (Fig 4, *K*) in the mouse groups described in Fig 4, *A*. **L–O,** Absolute numbers of lung Foxp3^{+}CD4^{+}Treg cells secreting IL-4 (Fig 4, *L*), IL13 (Fig 4, *M*), IL-17 (Fig 4, *N*) and IFN-γ (Fig 4, *O*) in the mouse groups described in panel Fig 4, *A*. Results are representative of 2 independent experiments. N=5 mice/group. *p<0.05, **<0.01, ***<0.001, ****<0.0001 by two-way ANOVA with post test analysis.

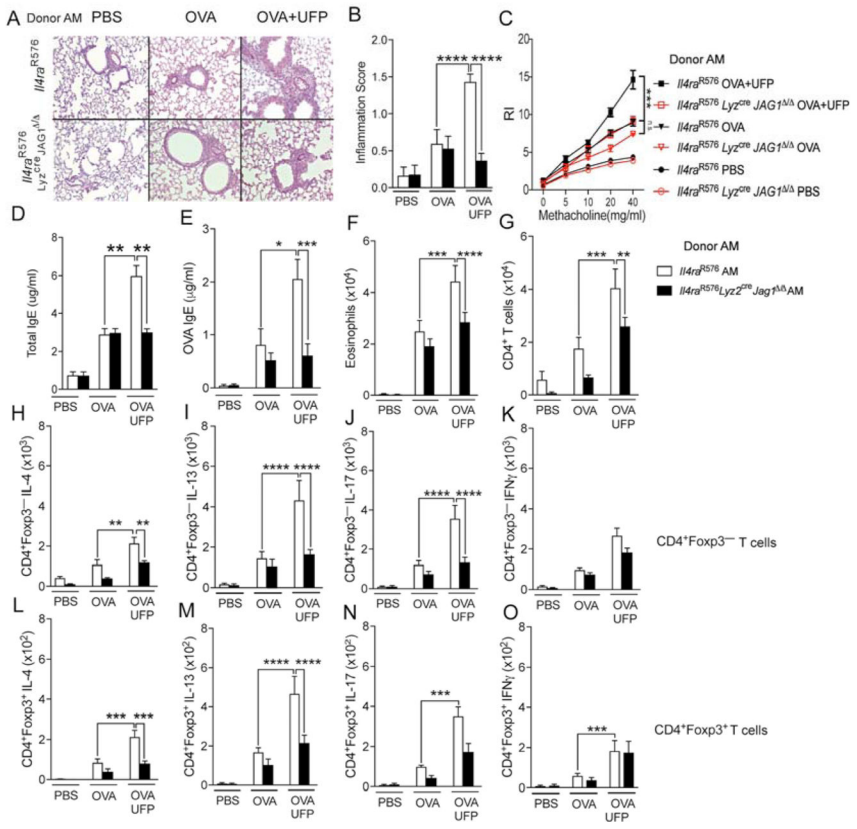

Fig. 5.

Jag1-sufficient AM rescue UFP-mediated allergic airway inflammation in *Il4ra*^{R576}*Lyz2*^{Cre}*Jag1*$^{\Delta/\Delta}$ mice. **A,** Representative PAS-stained sections of lung tissues of *Il4ra*^{R576}*Lyz2*^{Cre}*Jag1*$^{\Delta/\Delta}$ mice supplemented intra-tracheally with AM isolated from *Il4ra*^{R576} or *Il4ra*^{R576}*Lyz2*^{Cre}*Jag1*$^{\Delta/\Delta}$ mice that were either sham treated (PBS) or loaded with OVA323-339 peptide (OVA) alone or together with UFP (OVA+UFP). **B,** Inflammation scores of lung tissues of mice described in Fig 5, *A*. **C–G,** Airway hyper-responsiveness (Fig 5, *C*), numbers of eosinophils (Fig 5, *D*) and T cells (Fig 5, *E*) in the BAL fluids, total (Fig 5, *F*) and OVA-specific (Fig 5, *G*) levels in the sera of mice described in Fig 5, *A*. **H–K,** Numbers of lung Foxp3^{-}CD4^{+}T cells secreting IL-4 (Fig 5, *H*), IL13 (Fig 5, *I*), IL-17 (Fig 5, *J*) and IFN-γ (Fig 5, *K*) in the BAL fluids of mice described in Fig 5, *A*. **L–O,** Numbers of lung Foxp3^{+}CD4^{+}Treg cells secreting IL-4 (Fig 5, *L*), IL13 (Fig 5, *M*), IL-17 (Fig 5, *N*) and IFN-γ (Fig 5 *O*) in the BAL fluids of mice described in Fig 5, *A*. Results are representative of 3 independent experiments. N=7–12 mice/group. *p<0.05, **<0.01, ***<0.001, ****<0.0001 by two-way ANOVA with Bonferroni post test analysis. n.s.: not significant.

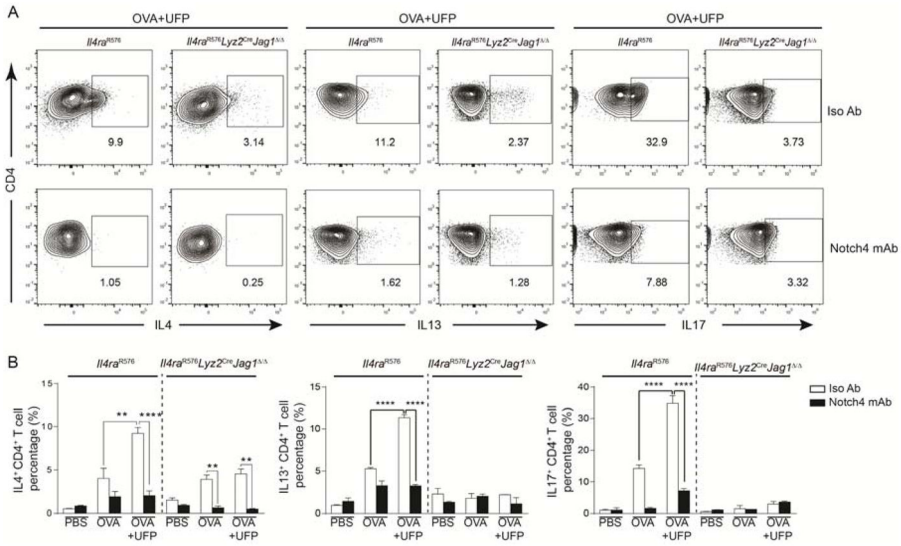

Fig. 6.

AM supports UFP-dependent upregulation of Th cell cytokine production by allergen-specific $CD4^+$ T cells in a Notch4-dependent manner. **A**, Representative flow cytometric analysis of IL-4, IL-13 and IL-17 cytokine production by naive *Il4ra*$^{R576}CD4^+DO11.10^+$ T cells co-cultured with FACS-purified AM isolated from *Il4ra*^{R576} or *Il4ra*^{R576}*Lyz2*^{Cre}*Jag1*$^{\Delta/\Delta}$ mice that have been pulsed with $OVA_{323-339}$ peptide in the presence of UFP (10 μg/mL). Co-cultures were treated with either isotype control (Iso) Ab or an anti-Notch4 mAb, as indicated, and cytokine analysis was carried out on gated $CD4^+Foxp3^-$ T cell. **B**, Frequencies of T cells expressing the respective cytokine upon co-culture with AM that have been either sham treated (PBS) or pulsed with $OVA_{323-339}$ peptide alone or in combination with UFP (10 μg/mL). Anti-Notch4 mAb or isotype control Ab were added as indicated. Results are representative of 3 independent experiments. *P < .05, **P < .01, ***P < .001, and ****P < .0001, two-way ANOVA with post-test analysis.

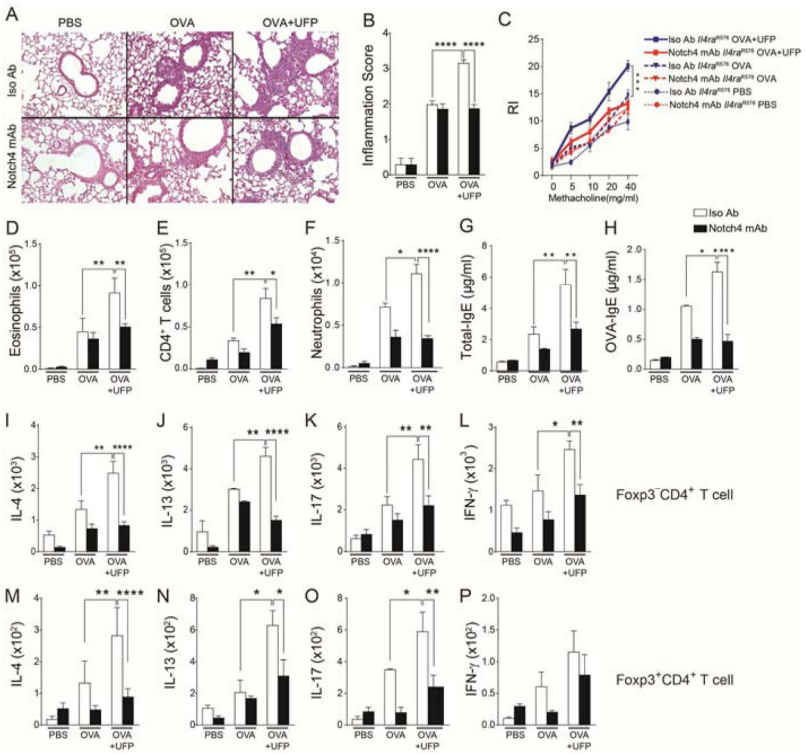

Fig. 7.

UFP enhances allergic airway inflammation in a Notch4-dependent manner. **A,** Representative PAS staining of lung tissues isolated from *Il4ra*^{R576} mice sensitized and challenged with OVA alone, or together with UFP, in the presence of either isotype control (Iso) Ab or an anti-Notch4 mAb. **B,** Inflammation scores in lung tissues of the mouse groups described in in Fig 7, *A*. **C–H** Airway hyper-responsiveness in response to methacholine (Fig 7, *C*), absolute numbers of eosinophils (Fig 7, *D*), T cells (Fig 7, *E*) and neutrophils (Fig 7, *F*) in the BAL fluids, total (Fig 7, *G*) and OVA-specific (Fig 7, *H*) levels in the serum of the mouse groups described in Fig 7, *A*. **I-L,** Absolute numbers of lung Foxp3^{-}CD4^{+}T cells secreting IL-4 (Fig 7, *I*), IL13 (Fig 7, *J*), IL-17 (Fig 7, *K*) and IFN-γ (Fig 7, *L*) in the mouse groups described in Fig 7, *A*. **M–P,** Absolute numbers of lung Foxp3^{+}CD4^{+}Treg cells secreting IL-4 (Fig 7, *M*), IL13 (Fig 7, *N*), IL-17 (Fig 7, *O*) and IFN-γ (Fig 7, *P*) in the mouse groups described in panel Fig 7, *A*. Results are representative of 2 independent experiments. N=5 mice/group. *p<0.05, **<0.01, ***<0.001, ****<0.0001 by two-way ANOVA with post test analysis.